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BRIEF REPORT

Diurnal variation of the startle reflex in relation to HPA-axis activity in humans

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Abstract

Diurnal variation of baseline startle amplitude was examined in 14 normal inpatients on a research unit where behavioral activity and environmental stimuli were highly controlled. We tested a hypothesized association between diurnal variations of salivary cortisol and reflex amplitude by recording acoustic startle eyeblinks shortly before bedtime, when cortisol was near its lowest daily level, and just after awakening, when cortisol was at its peak. Results showed that startle eyeblinks were greater during evening than morning sessions, whereas the opposite was true for cortisol levels. Skin conductance levels and reaction time performance also increased from morning to evening. These findings are consistent with accumulating evidence suggesting a possible link between startle reactivity and hypothalamic-pituitary-adrenal axis activity, and an association between diurnal variations in endogenous arousal and startle amplitude.

Descriptors: Startle, Circadian rhythm, Diurnal, Cortisol

The acoustic startle reflex is one of the most widely studied psychophysiological measures in the cognitive and affective sciences. Although considerable advances have been made in the understanding of attentional and emotional modification of startle, relatively few studies have focused on the neurobiological determinants of baseline startle amplitude, a metric of reactivity represented typically by the mean of all responses to a startling auditory stimulus recorded during an assessment. Because baseline startle is commonly employed as a dependent measure in infrahuman studies, it is a potentially valuable tool for efforts to extend, or translate, findings from animal studies to humans. The study of baseline startle is also important because it may inform our understanding of the clinical symptom of exaggerated startle in posttraumatic stress disorder. One line of research that may be relevant to the neurobiological determinants of this index of reactivity suggests that it may be influenced by hormones of the hypothalamo-pituitary-adrenal (HPA) axis. The purpose of this study was to further investigate this possibility by examining startle responses in normal volunteers at select intervals during the 24-h circadian cycle.

The HPA-axis is under a strong endogenous drive from the circadian timing system and therefore exhibits a pronounced daily rhythmicity. Its activity is initiated by the release of hypothalamic corticotropin-releasing hormone (CRH), which stimulates the secretion of adrenocorticotropic hormone from the anterior pituitary, which, in turn, triggers the production of cortisol by the adrenal gland. Cortisol serves a negative feedback function and shuts down HPA-axis activity by binding to corticosteroid receptors (glucocorticoid, mineralocorticoid) in the hypothalamus, pituitary, and extrahypothalamic regions (e.g., hippocampus). As a result, cortisol levels show a robust diurnal rhythm that is in phase opposition with CRH under normal conditions (i.e., in humans peaking around the time of awakening and dropping to their lowest levels around bedtime, whereas the opposite is true for CRH; Wong et al., 2000). In this study we took advantage of this naturally occurring rhythmicity to examine a hypothesized relationship between activity of the HPA-axis and baseline startle amplitude.

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Prior evidence for such an association comes from multiple sources. In rats, CRH administration produces a pronounced, dose-dependent enhancement of startle that can be blocked by pretreatment with a CRH receptor antagonist (Liang et al., 1992; Swerdlow, Britton, & Koob, 1989; Swerdlow, Geyer, Vale, & Koob, 1986). Antagonism of the glucocorticoid receptors, which produces a blockade of negative feedback, leads to an increase in startle amplitude (Korte, Korte-Bouws, Koob, deKloet, & Bohus, 1996) whereas corticosterone administration decreases it (Sandi, Venero, & Guaza, 1996). Similarly, administration of 20 mg oral hydrocortisone, which increases systemic levels of cortisol, has been shown to produce significant attenuation of baseline startle in healthy humans (Buchanan, Brechtel, Sollers, & Lovallo, 2001).

Additional evidence for this link comes from data showing that, in rats, the amplitude of the reflex exhibits a robust diurnal variation that is roughly opposite to the rhythm of corticosterone. Specifically, startle responses in nocturnal animals such as rats increase during the subjective night (i.e., the active phase; Chabot & Taylor, 1992a, 1992b; Horlington, 1970; Ison & Foss, 1997), with peak levels observed shortly before the onset of subjective day (i.e., the resting phase; Frankland & Ralph, 1995) whereas corticosterone levels decrease over the same interval (Seale, Wood, Atkinson, Harbuz, & Lightman, 2004). This diurnal rhythmicity of startle persists even in constant dark conditions (Chabot & Taylor, 1992a), suggesting that it is endogenously modulated by the circadian system, most likely via effectors of the central pacemaker such as the HPA-axis.[1]

To our knowledge, no prior study has examined the diurnal variation of baseline startle amplitude in humans or assessed concurrently the relation between indices of diurnal HPA-axis activity and startle. Indeed, research on the circadian influences on startle has advanced little since Chabot and Taylor (1992b) described the state of the knowledge on the mechanisms of the circadian control of startle as "an open question." Thus, to investigate a hypothesized relationship between the diurnal variation of cortisol and startle, we assessed acoustic startle eyeblink responses and concurrent salivary cortisol concentrations when cortisol was at its highest and lowest levels during waking hours (i.e., just after awakening and shortly before bedtime, respectively). The primary study hypothesis was that startle responses would be attenuated during morning sessions relative to evening sessions, presumably due to cortisol's negative feedback inhibition of CRH.

Method

Participants

Participants were 14 healthy men and women (5 men) between the ages of 19 and 33 years. Two were Hispanic, 1 was African-American, and the others were Caucasian. Exclusionary criteria included a history of mental illness including drug or alcohol dependency or recent substance abuse, endocrine disorder, use of medication known to influence the HPA-axis or melatonin secretion, pregnancy or inconsistent menstrual cycles, a history of night work in the preceding 3-year period or transmeridian travel (across >2 time zones) in the 3 months prior to the study, and circadian or sleep disorders.

Design

Time of day (i.e., "morning" vs. "evening") was the primary within-subject manipulation. The order in which participants underwent testing was counterbalanced (i.e., half of the participants completed the evening testing first; the other half underwent testing in the morning first). Two assessments were performed at each time of day.

Dependent Measures

Startle response. The startle stimulus consisted of a 50-ms burst of 104-dB white noise with immediate (<10 ms) rise time. The interval between probes ranged from 25 to 35 s with an average of 30 s. The stimulus was produced by a Coulbourn Instruments white noise generator (S81-02), amplified by an audio mixer-amplifier (S82-24) and presented binaurally through headphones. Startle eyeblinks were measured by recording EMG activity from Beckman miniature Ag/AgCl electrodes positioned over the orbicularis oculi muscle beneath the left eye. Coulbourn Instruments equipment was also used for signal amplification and processing. The raw EMG signal was amplified by a bioamplifier (S75-01) with high-pass and low-pass cutoff frequencies of 90 and 1000 Hz, respectively. The signal was then rectified and integrated with a contour-following integrator (S76-01) using a time constant setting of 20 ms. Digital sampling commenced at 1000 Hz, 50 ms before the onset of the startle probe to provide a baseline and continued for 150 ms after probe offset.

The startle response data were reduced off-line using a program developed by Curtin (1996) that scored startle-elicited blinks for magnitude and allowed scored responses to be visually inspected to control for artifacts before accepting them. A response was defined as the change from the mean EMG level during the 50-ms baseline period before the onset of the startle stimulus to peak of activity between 40 and 150 ms after probe onset. Trials were rejected if the mean EMG level during the baseline was greater than 10 μV or if EMG levels during the baseline period were unstable (i.e., showed more than 5 μV change). Data are presented in microvolt units.

Skin conductance level. Skin conductance level was sampled at 10 Hz and measured directly from an isolated skin conductance coupler (S71-23) using a constant 0.5-V output through 1-cm-diameter contact area Beckman surface Ag/AgCl electrodes. These were filled with 0.05 mol NaCL Unibase-saline paste and attached to adjacent sites on the hypothenar eminence of the nondominant hand. An index of tonic skin conductance level was computed by averaging across the entire 6 min of data collected during each assessment.

Salivary cortisol. Saliva samples were collected using a commercially available collection device ("salivette" by Sali-Saver). Cortisol levels were determined using the Coat-A-Count Cortisol Kit (DPC, Los Angeles, CA). The assay had a sensitivity of <0.01 μg/dL, a range of 0.01–5.0 μg/dL (0.1–50.0 ng/mL), with 4%–5% intra- and interassay coefficients of variation. Four of the 28 morning samples were returned with an insufficient quantity of specimen. These missing values were replaced with a repeat sample collected within 1 h of the missing one.

[1]These findings should not be confused with the phenomenon of "light-potentiated startle," a phasic reaction to bright light observed in rodents that is believed to reflect the unconditioned aversive properties of light exposure (cf. Walker & Davis, 1997).

Psychomotor Vigilance Task (Dinges & Powell, 1985). The Psychomotor Vigilance Task is a 10-min visual reaction time (RT) task and index of alertness. Participants are instructed to press a button as quickly as possible after the onset of a simple visual stimulus (i.e., the appearance of a millisecond counter in the center of the computer screen). Interstimulus intervals vary between 2 and 10 s. Mean RTs were computed by averaging responses across the 10-min task. Data were excluded for 1 participant who repeatedly fell asleep during the morning assessment.

Karolinska Sleepiness Scale (Åkerstedt & Gillberg, 1990). The Karolinska Sleepiness Scale is a simple, yet widely used and validated, 9-point scale of self-reported sleepiness (1 = *very alert*; 9 = *very sleepy, fighting sleep, difficulty staying awake*). Data for one observation were missing for 1 subject and replaced with the sample mean for that interval.

Procedure

All participants gave written informed consent and the study was conducted with the approval of the appropriate local human studies committees. The study took place in a private hospital room-like suite where participants lived 24 h a day with no access to the natural light–dark cycle or time cues. All behavioral activity (including dietary intake, motor activity, and sleep) and environmental stimuli (e.g., ambient light, temperature, and social interaction) were carefully controlled. Participants for this study were drawn from other ongoing inpatient circadian research protocols. Due to scheduling constraints imposed by those protocols, startle recordings took place on variable days relative to entry to the unit. All subjects entered the unit after maintaining a regular sleep/wake schedule for at least 7 days at home (verified via actigraphy). For 12 participants, startle recordings occurred on the second and/or third day of the inpatient stay. These were 24-h days with sleep episodes scheduled at the subjects' habitual sleep times. For the remaining 2 participants, startle recordings took place between days 19 and 22 of an inpatient protocol, after completing 5 regular 24-h days with consistent sleep/wake schedules. In all cases, measurements took place at a similar biological time (circadian phase), as participants had been entrained to a regular 24-h day for a sufficiently long time to synchronize the circadian system to that cycle. Eight out of the 9 female participants were assessed at the beginning of their menstrual phase when progesterone and estradiol were low, the 9th was assessed near the beginning of her follicular phase.

Physiological recording sessions took place at two intervals: during the first 2 h after awakening (morning) and during the 2 h before bedtime (evening). These windows offer the closest temporal approximations of cortisol's diurnal peak and nadir, respectively, that can be achieved during waking hours. The first morning assessment began an average of 14 min ($SD = 7$ min; range 7–34 min) after awakening, the second one 43 min after awakening ($SD = 12$; range 34–78 min) later. The first evening assessment began 90 min before bedtime ($SD = 34$ min; range 40–136 min), the second began 55 min before bedtime ($SD = 25$ min; range = 17–90 min). Subjects assigned to the morning first condition were reassessed during the evening of the same day; subjects in the evening first condition were reassessed after the subsequent sleep episode.

Each physiological recording was 6 min long and consisted of the presentation of 10 startle probes. To collect saliva, participants inserted a cotton swab ("salivette") into their mouths before each assessment and removed it afterwards. During the procedure, participants were either seated in a comfortable chair or in bed in a semirecumbent posture facing a computer monitor located approximately 1 m in front of them. They were instructed to sit still with their eyes open and to focus their gaze on a constant fixation point on the monitor throughout the procedure. Participants completed the Karolinska Sleepiness Scale and Psychomotor Vigilance Task within 30 min before or after the first assessment of each (morning or evening) session.

Data Analysis

Data were analyzed using multivariate analysis of variance (MANOVA) with repeated measures treated as variates. Each analysis included the two level within-subject factors Time (morning vs. evening) and Assessment (first vs. second) plus the between-subject factor Counterbalancing Order. Preliminary analyses revealed no significant main or interactive effects of participant Sex so this factor was eliminated from all subsequent analyses.

Results

Means and standard errors for the physiological measures are presented in Figure 1.

Startle Eyeblink Amplitude

Analysis of the startle response data revealed three significant effects: First, there was a main effect of Time, $F(1,12) = 12.70$, $p < .004$, partial $\eta^2 = .51$, with significantly larger startles during the evening than the morning. Second, there was a main effect of Assessment with larger startles during the first than second assessment, $F(1,12) = 17.41$, $p < .001$, partial $\eta^2 = .59$. Third, there was a Time × Assessment interaction, $F(1,12) = 4.97$, $p < .05$, partial $\eta^2 = .29$, indicating that the Assessment effect was greater during evening than morning sessions.

We also examined (a) the correlations between startle reflex amplitude and the other study measures at each time of day and (b) the diurnal covariation between startle reflex amplitude and the other study measures by computing diurnal change scores (i.e., p.m.–a.m. difference) and testing the correlation between change in each measure and change in startle. No significant associations were found in either analysis (range of r values: $\pm$ 0.04–0.32; range of p values: 0.91–0.29).

Salivary Cortisol

Cortisol analyses showed a main effect of Time, $F(1,12) = 87.63$, $p < .001$, partial $\eta^2 = .88$, indicating that levels were significantly higher during the morning than in the evening, and a Time × Assessment × Counterbalancing Order interaction $F(1,12) = 11.15$, $p < .006$, partial $\eta^2 = .48$. When this interaction was decomposed by examining the pattern of means for each counterbalancing group separately, analyses revealed that participants in the evening first group showed a significant Time × Assessment interaction, $F(1,6) = 14.03$, $p < .01$, partial $\eta^2 = .70$, reflecting an increase in cortisol levels from the first to the second assessment during the morning. This effect was nonsignificant in the morning first group.

Skin Conductance Level

Analyses of skin conductance levels revealed three effects of interest. First was a main effect of Time, $F(1,12) = 9.33$, $p < .01$,

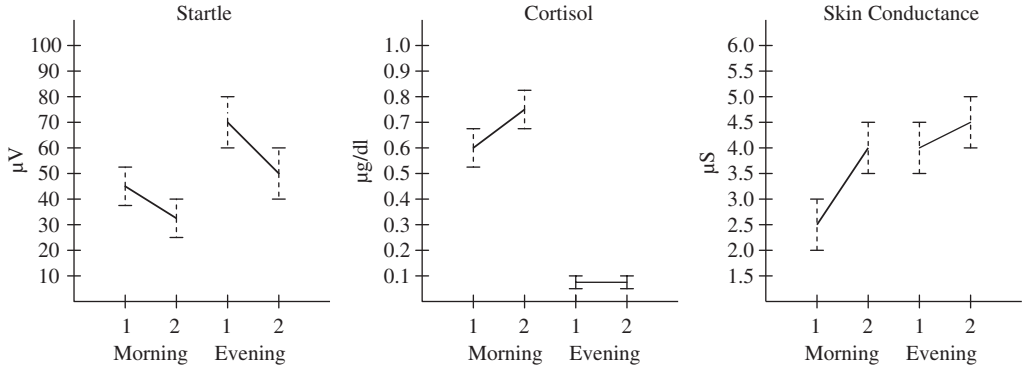

Figure 1. Mean levels and standard errors of physiological responses by time of day (morning vs. evening) and assessment (first vs. second).

partial $\eta^2 = .44$, with higher levels during the evening than in the morning. Second was a main effect of Assessment, $F(1,12) = 6.56$, $p < .03$, partial $\eta^2 = .35$, indicating that levels increased from the first to the second assessment. Third was a Time × Assessment × Counterbalancing Order interaction, $F(1,12) = 10.27$, $p < .008$, partial $\eta^2 = .46$. When this interaction was decomposed by examining the pattern of means for each counterbalancing group separately, analyses revealed a significant Time × Assessment interaction in the evening first group only, $F(1,6) = 31.89$, $p < .001$, partial $\eta^2 = .84$. This indicated that levels increased more from the first to second assessment in the morning compared to the evening in this group only.

Psychomotor and Alertness Measures

Analysis of the Psychomotor Vigilance Task data revealed a main effect of Time, $F(1,11) = 7.82$, $p < .02$, partial $\eta^2 = .42$, with RTs significantly shorter during the evening ($M = 280.35$ ms; $SD = 65.05$ ms) than morning sessions ($M = 323.13$ ms; $SD = 86.06$ ms). There were no significant differences between Karolinska Sleepiness Scale ratings obtained during morning ($M = 6.15$; $SD = 2.03$) versus evening sessions ($M = 5.71$; $SD = 1.59$).

Discussion

The primary finding of this study was that baseline startle amplitude was approximately 50% greater during the evening than in the morning. To our knowledge, this is the first report of diurnal variation of startle amplitude in humans—a phenomenon observed previously only in rats (Chabot & Taylor, 1992a, 1992b; Horlington, 1970; Ison & Foss, 1997). An important feature of the study was that it was conducted on an inpatient research unit where behavioral activity and environmental stimuli were carefully controlled throughout the day. This minimized the influence of potential confounds such as psychosocial stressors, physical activity, sleep, light intensity, temperature, posture, and dietary intake and lends support to our conclusion that the diurnal variation of startle amplitude is modulated endogenously by some aspect of the circadian timing system.

The finding that startle responses were smaller in the morning, when cortisol was near its diurnal peak, and larger in the evening, when cortisol was suppressed, is consistent with results of prior research suggesting a possible link between HPA-axis activity and baseline startle amplitude. Under normal conditions, cortisol levels show a robust diurnal rhythm that is in phase opposition with CRH, a well-established modulator of startle amplitude (Liang et al., 1992; Swerdlow et al., 1986, 1989). Cortisol levels peak around the time of awakening and drop to their lowest levels around bedtime, whereas the opposite is true for CRH (Wong et al., 2000). Thus, results of this study are in line with prior studies showing the potentiating effects of CRH on startle in rats and findings suggesting that pharmacologic increases of cortisol attenuate the startle response in humans, presumably via its inhibitory effect on CRH (Buchanan et al., 2001).

One important caveat to this conclusion is that skin conductance levels and performance on a vigilance task increased significantly from morning to evening, suggesting that tonic levels of arousal increased over the same interval. What might be driving observed changes in arousal and startle amplitude? One candidate system that has been implicated previously in both the circadian oscillation of arousal and as a determinant of startle amplitude is the brain's primary noradrenergic nucleus, the locus coeruleus (Aston-Jones, Chen, Zhu, & Oshinsky, 2001). Like the HPA-axis, the locus coeruleus is under a strong endogenous drive from the circadian timing system. As a result, levels of norepinephrine exhibit a pronounced daily rhythmicity. Evidence for a link to startle responding comes from studies showing that lesions of the locus coeruleus and drugs that inhibit its activity decrease startle reactivity (Adams & Geyer, 1981; Kehne & Davis, 1985), whereas drugs that increase locus coeruleus activity have the opposite effect (Davis, Redmond, & Baraban, 1979). Taken together, these findings raise the possibility that the diurnal variation of startle observed in this study reflects variation in activity of the locus coeruleus/norepinephrine system, as opposed to the HPA-axis. Importantly, this alternative is controverted by evidence that the circadian oscillations of norepinephrine and CRH are roughly in phase opposition in humans (i.e., norepinephrine in the cerebrospinal fluid is highest after awakening when CRH is lowest and vice versa; Wong et al., 2000). In other words, if the diurnal variation in startle amplitude were due to variation in levels of central norepinephrine, then startle should be elevated in the morning, when norepinephrine is elevated, rather than in the evening as was the case in this study. Thus, although the neurobiological mechanism underlying the diurnal variation of arousal and startle amplitude remains unclear, and it is possible that the perceived association between cortisol and startle is an epiphenomenon reflecting the influence of a third unmeasured and unknown factor, the findings of this

study are inconsistent with the alternative hypothesis that this effect can be attributed to activity of the locus coeruleus/norepinephrine system. Instead, when viewed in the context of the growing literature linking startle amplitude to CRH and cortisol levels, our results favor a possible link between HPA-axis activity and baseline startle amplitude.

Future investigations could directly examine the hypothesis that CRH mediates the association between diurnal variation in cortisol and changes in startle amplitude by employing a circadian procedure (such as constant routine) featuring repeated assessments of startle, cortisol, other HPA-axis parameters (adrenocorticotropic hormone and concentrations of CRH in cerebrospinal fluid) and additional indices of arousal throughout the 24-h day. Studies examining the impact of pharmacologic manipulations of HPA-axis parameters on startle amplitude would shed additional light on the hypothesized association between CRH level and startle amplitude. Research along these lines has the potential to further the knowledge of the relationship of HPA-axis activity to startle and may inform our understanding of the neurobiology of posttraumatic stress disorder, where the combination of exaggerated startle responses (Grillon & Morgan, 1999; Grillon, Morgan, Davis, & Southwick, 1998) and elevated CRH levels (Baker et al., 1999; Bremner et al., 1997) has been observed.

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